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13. Abstract *(Maximum 200 Words)* *(abstract should contain no proprietary or confidential information)*

Acute lung injury (ALI) activates tissue remodeling that is responsible for the excessive deposition and turnover of extracellular matrices. This project will explore the factors that control tissue remodeling in this setting by focusing on chronic ethanol ingestion, a factor that renders the lung susceptible to ALI. We hypothesize that chronic ethanol ingestion renders the lung susceptible to ALI by acting on α7 nicotinic acetylcholine receptors (α7 nAChRs) and stimulating the expression of tissue remodeling genes such as fibronectin (FN). Aberrant deposition of FN affects the structure of the lung and promotes a "proinflammatory state" that drives the development of ALI after infection. The specific aims are to: 1) Determine the role of α7 nAChRs in ethanol induction of FN. 2) Delineate the intracellular pathways responsible for the induction of FN in fibroblasts in response to ethanol. 3) Elucidate the effects of ethanol-induced FN expression on lung cell function. 4) Study ethanol-induced FN expression in a rat model of sepsis-induced ALI. Service men and women are exposed to conditions considered risk factors for ALI (e.g., trauma, toxic gas, infection). Tissue remodeling is considered key to the development of the irreversible consequences of ALI.

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I. INTRODUCTION

Acute lung injury is a major cause of morbidity and mortality in the U.S. Its most dramatic manifestation is the Acute Respiratory Distress Syndrome or ARDS, an illness that affects over 150,000 Americans each year and that leads to death in up to 40-50% of cases (1). Acute lung injury is characterized by the activation of tissue remodeling which is responsible for the excessive deposition and turnover of extracellular matrices (2). Ultimately, it is the ability of the host to control tissue remodeling that determines the final outcome in acute lung injury (2,3). Although external factors capable of eliciting acute lung injury have been identified (e.g., infection, trauma), little is known about the factors that control the tissue remodeling response. This project addresses this very aspect. It was prompted by an intriguing report published in 1996 linking chronic ethanol ingestion to outcomes in ARDS (4). This report identified ethanol as an independent outcome variable in ARDS, a finding that is considered one of the most significant observations made in the area of acute lung injury. Today, it is believed that the development of acute lung injury is related to chronic ethanol ingestion in over 50% of cases (5). Because of its importance, we began to explore the mechanisms by which ethanol affects tissue remodeling and predisposes the lung to acute lung injury. Preliminary observations made in this area led us to hypothesize that ethanol ingestion renders the lung susceptible to acute lung injury by acting on $\alpha 7$ nicotinic acetylcholine receptors (nAChRs) expressed by fibroblasts, and stimulating their expression of tissue remodeling genes; in particular that of fibronectin (FN). The consequent aberrant deposition of FN in the lung parenchyma induces the expression of potent transcription factors (e.g., AP-1, NFkB) in macrophages and other cells that come in contact with the newly deposited fibronectin-containing matrix. This promotes a "proinflammatory state" that primes resident and incoming immune cells recruited by diverse pulmonary insults (e.g. infection) thereby amplifying inflammatory responses in the lung that promote the development of acute lung injury. The following objectives were designed to address the hypothesis:

Objective I. **Determine the role of $\alpha 7$ nAChRs (and perhaps other nAChRs) in ethanol induction of FN.**

Objective II. **Delineate the intracellular pathways responsible for the induction of FN in fibroblasts in response to ethanol.**

Objective III. **Elucidate the effects of ethanol-induced FN expression on cytokine expression.**

Objective IV. **Study the effects of ethanol-induced FN expression in the rat model of sepsis-induced acute lung injury.**

II. BODY

This work has led to important observations that are described below under each of the objectives proposed in the initial application:

Objective I. **Determine the role of α7 nAChRs (and perhaps other nAChRs) in ethanol induction of FN.**

Ethanol has been shown to act on nicotinic acetylcholine receptors (nAChRs) in neuronal cells. Because nAChRs have been detected in NIH3T3 fibroblasts and monkey lung fibroblasts, among other non-neuronal cells, we examined the role of these receptors in our system. First, we demonstrated that NIH3T3 cells express mRNA coding for α7 nAChRs. Of note, the α7 mRNA was increased after ethanol stimulation. Further evidence for the presence of α7 nAChRs was derived from α-bungarotoxin- (α-BGT) binding assays. α-BGT is a specific competitive ligand for α7 nAChRs. Consistent with the expression of α7 nAChRs, we found binding sites for α-BGT on the surface of fibroblasts. As expected, the binding of α-BGT was increased after the exposure of the cells to ethanol for 24 hours. This increase in ethanol induced α-BGT binding was decreased by excess unlabeled α-BGT and ethanol. Together, these observations indicated that fibroblasts express α7 mRNA and have α7 nAChR protein on their surface. To confirm a role for α7 nAChRs in the ethanol-induced fibronectin response, we pretreated fibroblasts with α-BGT prior to exposing them to ethanol. α-BGT completely prevented the expression of the fibronectin in response to ethanol.

Objective II. Delineate the intracellular pathways responsible for the induction of FN in fibroblasts in response to ethanol.

II.a. Ethanol induces fibronectin gene transcription

Because of the increased deposition of fibronectin around fibroblasts in the lungs of ethanol-exposed rats, we explored the effects of ethanol in cultured lung fibroblasts with the intention of developing an *in vitro* model of ethanol-induced fibronectin expression. Using RT-PCR, we found that ethanol induced the expression of endogenous fibronectin mRNA in primary rat lung fibroblasts. This coincided with an increase in fibronectin protein production as determined by western blotting. These observations identify the lung fibroblast as a potential target for ethanol in the lung.

The data presented above also suggested that the effects of ethanol on fibronectin expression occur at the level of gene transcription. To test this, we used NIH3T3 fibroblasts which were considered a suitable model for our studies because they respond to ethanol in a similar fashion as primary lung fibroblasts. NIH3T3 cells treated with ethanol (60 mM) show increased accumulation of endogenous fibronectin mRNA as determined by RT-PCR. The increase in fibronectin mRNA was detectable as early as 8 hours after ethanol stimulation. As expected, the ethanol-induced response was associated with a subsequent increase in fibronectin protein production as determined by western blotting that was highest at 48 hours.

When the transcription of the gene was tested in NIH 3T3 cells stably transfected with a construct containing the human fibronectin promoter fused to a luciferase reporter gene, pFN(1.2kb)LUC, as previously reported (20), we found that ethanol increased the transcription of pFN(1.2kb)LUC when compared to control. As before, the effects of ethanol were both time-dependent (peaking around 8 hours) and dose-dependent (optimal at 60 mM). At the concentrations used, ethanol had no effect on cell viability (not shown).

II.b. Ethanol-induction of fibronectin gene transcription is dependent on protein kinase activity and the induction of cAMP Response Element Binding Protein (CREB)

The stimulation of fibronectin by serum and other agents has been shown to be preceded by protein kinase activation and phosphorylation of the transcription factor cAMP Response Element Binding Protein (CREB), followed by CREB binding to cAMP response elements (CREs) in the fibronectin promoter. To test whether this pathway also mediates the ethanol-induced fibronectin response, we performed the following experiments. First, we tested the effects of protein kinase inhibitors on the ethanol-induced response. A protein kinase C inhibitor, Calphostin C, abolished the constitutive expression of fibronectin as well as expression induced by ethanol. PD98059, an inhibitor of MEK-1 (26), which is upstream of erk-1 and erk-2 in the Mitogen Activation Protein Kinase pathway, also inhibited the ethanol-induced fibronectin response. The inhibitory effects of these agents at the concentrations used were not associated with increased cell death (not shown).

Second, we tested the effects of ethanol on CREB. The exposure of fibroblasts to 60 mM ethanol induced the phosphorylation of CREB in a time-dependent fashion. This event was associated with increased binding of CREB to DNA as determined by EMSA. Note that a competing oligonucleotide abolished the binding of CREB, whereas the mutated oligonucleotide did not affect it.

II.c. Ethanol induction of fibronectin gene transcription is dependent on specific promoter elements.

The above studies suggested a role for specific promoter elements within the fibronectin gene, namely the CREs, in the ethanol-induced effect. To confirm this role, we tested fibroblasts transfected with deletion constructs of the fibronectin gene promoter. All previous experiments were performed using pFN(1.2kb)LUC. We tested cells transfected with pFN(0.5kb)LUC, that lacks most of the 5' sequences present in pFN(1.2kb)LUC, but contains the 3 CREs, or transfected with pFN(0.2kb)LUC, that lacks all three CREs. Ethanol stimulated the transcription of pFN(1.2kb)LUC over control as before. The stimulatory effect of ethanol was unaffected when the deletion construct pFN(0.5kb)LUC was tested indicating that 5' sequences proximal to the CREs are not needed for optimal stimulation. In contrast, no stimulation was noted for ethanol when all CREs were lacking.

These data suggest, but do not prove, a role for CREs in the ethanol-induced response. To strengthen the association between CREs and the response tested, we measured fibronectin expression in cells exposed to 60 mM ethanol after transfection with a competing consensus CRE oligonucleotide. The consensus CRE oligonucleotide greatly diminished the ethanol-induced fibronectin response, whereas the control mutated CRE oligonucleotide had no effect.

Objective III. Elucidate the effects of ethanol-induced FN expression on cytokine expression.

We have not begun experiments in this area.

Objective IV. **Study the effects of ethanol-induced FN expression in the rat model of sepsis-induced acute lung injury.**

We have not begun experiments in the animal model of sepsis-induced acute lung injury. However, we have investigated the effects of ethanol alone on fibronectin expression in lung *in vivo*. To examine the effects of ethanol on fibronectin expression in lung, rats were fed the Lieber-DeCarli isocaloric liquid diet that contains 36% of total calories provided as ethanol. This diet was previously shown to increase endotoxin-mediated acute edematous injury in rat lungs isolated and perfused *ex-vivo*. After 6 weeks, the lungs of control rats and rats fed with ethanol diets were harvested and processed for the detection of fibronectin mRNA (by RT-PCR) and protein (by immunohistochemistry). The lungs of rats fed with ethanol showed increased fibronectin mRNA content when compared to pair-fed control animals. As expected, immunohistochemical analysis of control lungs showed fibronectin deposition in vascular, airway structures, and within alveolar septae. However, experimental lungs showed increased fibronectin deposition as demonstrated by intense staining of lung structures including the alveolar septae; both epithelial cells and interstitial fibroblasts appeared stained. These observations demonstrate that chronic ethanol ingestion in rats is associated with increased expression and deposition of the matrix glycoprotein fibronectin *in vivo*.

III. KEY RESEARCH ACCOMPLISHMENTS

- We demonstrated that transformed and primary lung fibroblasts express functional $\alpha 7$ nAChRs.
- We showed that exposure of these cells to ethanol triggers a cascade of intracellular signaling events that stimulate the transcription of the gene that codes for the matrix glycoprotein fibronectin. Many of these intracellular events were delineated.
- We demonstrated that these events tested *in vitro* appear to apply to the situation *in vivo* since rats chronically fed with ethanol showed increased expression of fibronectin in their lungs when compared to their control counterparts.

IV. REPORTABLE OUTCOMES

- **Abstracts**

Roman, J, Ritzenthaler, JD, Roser, S, Bechara, R, Guidot, DM. Ethanol-induced fibronectin expression in lung fibroblasts: Implications for ARDS. *Am J Respir Crit Care Med*, 165:A365, 2002.

- **Review article published**

Guidot, DM and **Roman, J**. Chronic ethanol ingestion increases susceptibility to acute lung injury: role of oxidative stress and tissue remodeling. Requested Review. Chest 122:309S-3014S, 2002.

- **Manuscript in press or submitted for publication in peer-reviewed journals**

Bechara RI, Brown LAS, Eaton DC, **Roman J**, Guidot DM. AT2 receptor expression and apoptosis in alveolar epithelial cells of ethanol-fed rats. *Alcohol Clin Exp Res*, accepted for publication.

Roman J, Ritzenthaler JD, Bechara B, Brown LA, **Guidot DM**. Ethanol stimulates the expression of fibronectin in lung fibroblasts via nicotinic acetylcholine receptor-dependent signals. J Clin Invest , submitted.

- **Presentations**

June 2003 Visiting Professor, University of South California; Lecture: Tissue remodeling in the lung – Control of fibronectin gene expression.

Nov 2002 Speaker, Regulation of fibronectin expression in lung fibroblasts. Department of Medicine Research Conference, Emory University, Atlanta, GA.

V. CONCLUSIONS

Together, these data unveil a novel mechanism by which ethanol can promote lung tissue remodeling, and how this effect can promote the development of acute lung injury under the right circumstances. In addition, it identifies the lung fibroblast as a target for alcohol and a likely candidate for driving tissue remodeling in the lung of chronic alcoholics. The work also helps establish a rat model of chronic ethanol ingestion that can be used for further investigations related to lung pathophysiology including those proposed here related to sepsis. The next phase of this work will address the consequences of excess fibronectin expression in lung using in vitro and in vivo models proposed in Objectives III and IV.

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Ethanol stimulates the expression of fibronectin in lung fibroblasts via nicotinic acetylcholine receptor-dependent signals

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ABSTRACT

Ethanol renders the lung susceptible to acute lung injury in the setting of insults such as sepsis. The mechanisms mediating this effect are unknown, but activation of tissue remodeling is considered key to this process. We found that chronic ethanol ingestion in rats increased the expression of fibronectin, a matrix glycoprotein implicated in acute lung injury. In cultured transformed and primary rat lung fibroblasts, ethanol induced fibronectin mRNA and protein expression in a dose- and time-dependent fashion. The effect of ethanol was prevented by inhibitors of protein kinase C and mitogen-activated protein kinases, and was associated with the phosphorylation and increased DNA binding of the transcription factor CREB followed by increased transcription of the fibronectin gene. Fibroblasts were found to express α7 nicotinic acetylcholine receptor (nAChR) and ethanol induction of fibronectin was abolished by α-bungarotoxin, a specific inhibitor of α7 nAChRs. The ethanol-induced fibronectin response was dependent on ethanol metabolism since 4-methypyrazole, an inhibitor of alcohol dehydrogenase, abolished the effect. These observations suggest that ethanol stimulates lung fibroblasts to produce fibronectin by inducing specific signals transmitted via α7 nAChRs, and that this might represent a mechanism by which ethanol renders the lung susceptible to acute lung injury.

Key words: extracellular matrix, tissue remodeling, signal transduction, gene transcription, nicotinic acetylcholine receptors, lung injury

INTRODUCTION

The Acute Respiratory Distress Syndrome (ARDS) is a devastating disease that afflicts approximately 75,000-150,000 individuals per year in the United States (1). The most common at-risk diagnoses associated with the development of ARDS are sepsis, trauma, and the aspiration of gastric contents. The mechanisms that lead to the development of this syndrome in some patients and not others are unknown, but a recent discovery points to alcohol abuse as an important predisposing factor. This association was first identified by the work of Moss and colleagues who demonstrated that chronic alcohol abuse in humans independently increases the incidence of ARDS in at-risk patients, and is associated with increased mortality related to multi-organ failure (2).

Ethanol also predisposes rats to edematous lung injury elicited by endotoxemia or sepsis thereby mimicking the human condition (3). The use of this model has greatly improved our undertstanding of the cellular mechanisms responsible for the effects of ethanol in the lung. The data available to date indicate that chronic (6-8 weeks) ingestion of ethanol in these animals results in decreased levels of glutathione, an important antioxidant in the lung (3,4). This defect is associated with alterations in epithelial cell permeability (5), decreased alveolar liquid clearance (6), decreased cell viability (7), and decreased surfactant production (8). Alterations in glutathione metabolism have also been confirmed in humans that abuse alcohol (9).

Studies performed in rats chronically fed with ethanol also revealed activation of tissue remodeling in the lung. In particular, ethanol induced activation of matrix-degrading enzymes of the matrix metalloproteinase family (10), and increased the production of the pro-fibrotic factor Transforming Growth Factor β1 (11). These studies suggest that activation of tissue remodeling, with subsequent alterations in extracellular matrix expression, deposition and degradation, might represent another mechanism by which ethanol can affect the lung and render it susceptible to acute lung injury (12).

More recently, we found that chonic ethanol ingestion also increases the expression of fibronectin in the lung. This multidomain cell-adhesive glycoprotein is increased in acute lung injury and its production is elicited experimentally by agents associated with this illness (e.g., paraquat) (13,14). Although the exact role of fibronectin in lung is unknown, its ability to promote matrix deposition and coagulation, and to induce the migration and activation of

inflammatory cells *in vitro*, among other functions, suggest that fibronectin is not only a sensitive marker of injury, but that it is a key player in the pathogenesis of acute lung injury (15). Accordingly, this report explores the intracellular mechanisms that mediate ethanol-induced fibronectin expression in fibroblasts in order to gain insight into the pathways involved in activation of tissue remodeling in the lungs of experimental animals exposed to ethanol chronically.

METHODS

Experimental Reagents

α-bungarotoxin was purchased from Amersham Biosciences, Piscataway, NJ. The anti-CREB antibody, anti-phospho-CREB antibody, and Mitogen Enhanced Kinase-1 (MEK1) inhibitor PD98059 were purchased from Cell Signaling Technology, Inc., Beverly, MA. Ethanol, dbcAMP, Calphostin C, and 4-methylpyrazole were purchased from Sigma Chemical Company, St. Louis, MO. All other reagents were purchased from Fisher Scientific (Pittsburgh, PA) or Sigma Chemical Company (St. Louis, MO) unless otherwise specified.

Cell Culture and Treatment

Murine NIH3T3 fibroblasts were maintained in Dulbecco's Modified Eagle's Medium (DMEM) with 4.5 gm/l glucose supplemented with 10% heat inactivated fetal bovine serum (FBS), 1% antibiotic-antimycotic solution (100 units/ml penicillin G sodium, 100 units/ml streptomycin, 0.25 ug/ml amphotericin B), and incubated in a humidified 5% CO_2 incubator at 37°C. The cells were harvested by trypsinization with 2.5 X trypsin and 5.3 mM EDTA, washed with PBS, counted, and plated at 1.5×10^5 cells/ml in 12 well tissue culture dishes in 10% FBS. Concurrently, cells were treated with calphostin C (1×10^{-7} M) or MAPK inhibitor (50 uM). The doses of experimental agents were chosen based on optimal doses reported in the literature. Cells were harvested at 24 hr.

Primary lung fibroblasts were obtained from harvested rat lungs by discarding the outer 3 mm of lung periphery and cutting the remaining lung parenchyma tissue into 1 mm sections. Tissue sections were washed twice in sterile PBS, resuspended in Dulbecco's Modified Eagle's Medium (DMEM) with 4.5 gm/l glucose supplemented with 10% fetal bovine serum (FBS), 1% antibiotic-antimycotic solution (100 units/ml penicillin G sodium, 100 units/ml streptomycin, 0.25 ug/ml amphotericin B), transferred to a tissue culture dish and incubated in a humidified 5% CO_2 incubator at 37°C for 1-3 wks to allow fibroblasts to migrate out of tissue sections. Primary lung fibroblasts were between 3-5 passages when used in experiments.

Detection of fibronectin and CREB phosphorylation by western blot

Fibroblasts were treated with 60 mM ethanol for 0-48 hr, washed with ice-cold PBS, and lysed in 1 ml of homogenization buffer (50 mM NaCl, 50 mM NaF, 50 mM NaP_2O_7-10 H_20, 5 mM EDTA, 5 mM EGTA, 2 mM Na_3 $V0_4$, 0.5 mM PMSF, 0.01% Triton X-100, 10 ug/ml leupeptin, 10 mM HEPES, pH 7.4) by repeated passages through a 26 gauge needle. The resulting homogenate was centrifuged at 14,000 rpm for 5 min. at 4°C. Protein concentration was determined by the Bradford method. The protein (100 ug) was mixed with an equal volume of 2X sample buffer (125 mM TrisHCL, pH 6.8, 4% SDS, 20% glycerol, 5-10% β-mercaptoethanol, 0.004% bromophenol blue), boiled for 5 min., loaded onto a 10% SDS-polyacrylamide gel with a 3.9% stacking gel, and electrophoresed for 1 hr at 60 mA. The separated proteins were transferred onto nitrocellulose using a BioRad Trans Blot semi-dry transfer apparatus for 30 min. at 25 mA, blocked with blotto (1x TBS (10 mM Tris-HCL, pH 8.0, 150 mM NaCl), 5% non-fat dry milk, 0.05% Tween-20) for 1 hr at room temperature, and washed twice for 5 min. with wash buffer (1x TBS, 0.05% Tween-20). Blots were incubated with a polyclonal antibody raised against bovine fibronectin (antibody 1.2; 1:500 dilution) for 2 hr at 4^0C, washed 3 times for 5 min. with wash buffer, and incubated with a secondary donkey antibody raised against rabbit IgG conjugated to horse radish peroxidase (1:10,000 dilution) for 1 hr at room temperature. Endogenous levels of CREB activated by phosphorylation at Ser 133 were detected using an antibody specific for phosphor-CREB (1:1000 dilution) for 2 hr at 4^0C, washed 3 times for 5 min. with wash buffer, and incubated with a secondary goat antibody raised against rabbit IgG conjugated to horse radish peroxidase (1:5000 dilution) for 1 hr at room temperature. The blots were washed 4 times in wash buffer, transferred to freshly made ECL solution (Amersham, Arlington, IL) for 1 min., and exposed to X-ray film. Protein bands were quantified by densitometric scanning using a GS-800 Calibrated laser densitometer (Bio-Rad, Hercules, CA).

Detection of mRNAs by reverse transcriptase-polymerase chain reaction

Fibroblasts were exposed to ethanol (0-100 mM) and tested at 2, 6, 8, and 24 hr for various mRNAs using an RT-PCR Bioluminescence Assay. The procedure for bioluminescent detection of mRNA was performed as previously described (16,17). Amplification of PCR products was achieved using 5'-biotinylated (forward) primers; the 3' primers were not modified,

and the PCR products ranged in size from 300 to 350 base pairs. Cycled curve studies were performed to ensure that for the amounts of cDNA being amplified, the reaction had not reached plateau of the amplification curve at a constant number of cycles for any primer pair. Negative controls consisted of dH_20 and RNA without RT-PCR products, and standardization was made to the housekeeping gene β-actin or HPRT (hypoxanthine phosphoribosyltransferase). The biotinylated primer-PCR product was captured on streptavidin-coated plates (Boehringer Mannheim) and probed with DIG labeled probes. The oligos were DIG-labeled using the DIG Oligonucleotide Tailing Kit plates (Boehringer Mannheim, Indianapolis, IN). Anti-digoxigenin antibody labeled with the bioluminescent molecule aequorin (AquaLite®, SeaLite Sciences, Bogart, GA) was added and luminescence measured on a Labsystems Luminoskan Ascent Plate Luminometer after triggering with calcium. Because of its semi-quantitative nature, the relative amounts of a specific mRNA were compared to one another within the same experiment. All products were verified by agarose gel electrophoresis to ensure that the predicted mRNA species was being examined.

Primers and probes for RT-PCR reactions were based on GenBank published sequences and are as follows: rat FN forward primer (AGAGCATACCTCTCAGAG), rat fibronectin reverse primer (CTGCTCATCAGTTGGGAA), rat fibronectin probe (TCTATCACCCTCACCAAC); rat HPRT forward primer (GTCATGAAGGAGATGGGA) rat HPRT reverse primer (CAGCAA GCTTGCACCCTT), rat HPRT probe (GCTTGACCAAGGAAAGCA); rat nAChR α7 forward primer (ACGAGGTTGCGCTTTGAG), rat nAChR α7 reverse primer (AATCCG GTGGCTTGATGC), rat nAChR α7 probe (ATTCTGGGCGCAGGACTC); mouse fibronectin forward primer (CTGTGACAACTGCCGTAG), mouse fibronectin reverse primer (CAGCTTCTCCAA GCATCG), mouse fibronectin probe (ACCAAGGTCAATCCACAC); mouse β-actin forward primer (ATGGATGACGATATCGCT), mouse β-actin reverse primer (ATGAGGTAGTCTGTCAGG T), mouse β-actin probe (GGATGGCTACGTACATGGCT); mouse nAChR α7 forward primer (GTAACCATGCGCCGTAGG), mouse nAChR α7 reverse primer (CCGAGGCTT GTGCTGAC), mouse nAChR α7 probe (GGTGCTGGCGAAGTACTG).

^{125}I-α-BGT-binding assay.

The α-BGT binding assay was performed using the method of Breese et al. (18,19) to detect nicotinic acetylcholine receptors on the surface of fibroblasts. Fibroblasts (1 x 10^6) were incubated with 5 nM [^{125}I]Tyr^{54} α-BGT (specific activity 2,000 Ci/mM) alone or with 2 mM nicotine for 4 hr at 37°C/5% $C0_2$. Control cells were incubated with binding buffer (TBS + 0.2% BSA) or binding buffer with 2 mM nicotine for nonspecific binding for 1 hr at room temperature. The cells were rinsed twice in binding buffer at 37^{0}C for 5 min., followed by three washes in TBS for 15 min., and one wash in PBS for 5 min. Afterwards, ^{125}I radioactivity bound to the functional nAChRs contained in the samples was quantified by gamma counter.

Examination of fibronectin gene transcription

To evaluate for fibronectin gene transcription, the pFN(1.2kb)LUC promoter construct was introduced into murine NIH3T3 fibroblasts from the American Type Culture Collection (CRL #1658; Rockville, MD) via electroporation to create stable transfectants (20). pFN(1.2kb)LUC contains approximately 1200 base pairs (bp) of the 5' flanking region of the human fibronectin gene isolated from the human fibrosarcoma cell line HT1080. This construct includes 69 bp of exon 1, a CAAT site located at -150 bp, and the sequence ATATAA at -25 bp from the transcription start site. It also contains several previously identified regulatory elements such as three cyclic AMP (cAMP) response elements located at -415 bp, -270 bp, and -170 bp, and an SP-1 site at -102 bp from the transcription start site. The promoter was subcloned into the sma I site of pGL3 Basic Luciferase Reporter Vector (Promega, Madison, WI) (20).

The transfected NIH3T3 fibroblasts were maintained in Dulbecco's Modified Eagle's Medium (DMEM) with 4.5 gm/l glucose supplemented with 10% heat inactivated fetal bovine serum (FBS), 1% antibiotic-antimycotic solution (100 units/ml penicillin G sodium, 100 units/ml streptomycin, 0.25 ug/ml amphotericin B), and incubated in a humidified 5% CO_2 incubator at 37°C. The cells were harvested by trypsinization with 2.5X trypsin and 5.3 mM EDTA (Sigma Chemical Company, St. Louis, MO), washed with PBS, counted, and plated at 1.5 x 10^5 cells/ml in 12 well tissue culture dishes in 10% FBS. Concurrently, cells were treated with ethanol (0-160 mM) for various periods of time. Afterwards, the cells were tested for luciferase activity. For this, the cells were harvested by cell scraper, washed with PBS, resuspended in 100 ul of cell lysis buffer (Promega, Madison, WI), sonicated, and a 10 ul aliquot was tested by adding 50 ul Luciferase Assay Reagent (Promega, Madison, WI). Light intensity was measured using a

Labsystems Luminoskan Ascent Plate Luminometer. Results were recorded as normalized luciferase units and adjusted for total protein content that was measured using the Bradford Method (21).

Electrophoretic DNA Mobility Shift Assay (EMSA)

Fibroblasts (3 x 10^6) were seeded onto 150 mm^2 tissue culture flasks and incubated in 10% FBS for 24 hr with and without concurrent treatment with ethanol at the doses described above. Cells were washed with ice cold PBS, and nuclear binding proteins were extracted by a published method (22). Protein concentration was determined by the Bradford method using BioRad protein assay reagent (21). Double-stranded cAMP-responsive element binding protein (CREB) consensus oligonucleotide (5'AGAGATTGCCTGACGTCAGAGAGCTAG) was labeled with biotin-N4-CTP using terminal deoxynucleotidyl transferase (TdT) enzyme. Nuclear protein (5 ug) was incubated with biotin-labeled double-stranded CREB for 20 min. at room temperature as described previously (20). For competition reactions, non-biotin labeled consensus and mutated CREB double-stranded oligonucleotides (5'AGAGATTGCCTGTGGTC AGAGAGCTAG) were added to the reaction mixture at 50X molar concentration. DNA-protein complexes were separated on 6% native polyacrylamide gel (20:1 acrylamide/bis ratio) in low ionic strength buffer (22.25 mM Tris borate, 22.25 mM boric acid, 500 mM EDTA) for 2-3 hr at 4°C at 10V/cm^2. DNA and DNA-protein complexes were transferred to nylon membrane using a BioRad Trans Blot semi-dry transfer apparatus for 1 hr at 25 v. and crosslinked using the Fb-UVXL-1000 UV Crosslinker (Fisher Scientific, Pittsburgh, PA). DNA and DNA-protein complexes were detected using a Streptavidin-Horseradish Peroxidase Conjugate and Lightshift™ Chemiluminescent Substrate according the manufacturer's instructions (Pierce Biotechnology, Rockford, IL). The membrane was exposed to X-ray film for 1 min.

Screening for Lipopolysaccharide (LPS)

Experimental reagents were reconstituted in LPS-free water (Sigma, St. Louis, MO). All treatment materials and culture media were screened with a limulus based endotoxin assay with a sensitivity of 0.06 ng/ml (Endotect-Schwarz/Mann Biotech, Cleveland, OH) as described (23,24). Reagents were found to remain endotoxin-free throughout all experiments.

Animal model of chronic ethanol ingestion and lung immunohistochemistry

The animal model of chronic ethanol ingestion has been described previously (3-8,10). Briefly, young adult male Sprague-Dawley rats (200-250 g) were fed the Lieber- DeCarli liquid diet (Research Diets, new Brunswick, NH) containing either ethanol (36% total calories) or the isocaloric carbohydrate substitution with Maltin-Destrin (control diet). The diets are otherwise identical in protein, lipid, and essential nutrient composition. This is a standard experimental diet in ethanol ingestion models and we have used it extensively. During the first two weeks of the dietary regimen, the ethanol-fed rats were gradually acclimated to the ethanol, receiving 12% of their total calories as ethanol (1/3 strength) for one week, then 24% of their total calories as ethanol (2/3 strength) for one week, and then full-strength diet (36% of total calories as ethanol) for four weeks, for a total of six weeks of ethanol ingestion. Afterwards, the animals were euthanized followed by the isolation of the lungs for RNA isolation (see above) and immunohistochemistry. Control and experimental lungs were processed and submitted to immunohistochemistry with an anti-fibronectin antibody as previously described (24).

RESULTS

Ethanol increases fibronectin expression and deposition in rat lungs

To examine the effects of ethanol on fibronectin expression in lung, rats were fed the Lieber-DeCarli isocaloric liquid diet that contains 36% of total calories provided as ethanol. This diet was previously shown to increase endotoxin-mediated acute edematous injury in rat lungs isolated and perfused *ex-vivo* (3). After 6 weeks, the lungs of control rats and rats fed with ethanol diets were harvested and processed for the detection of fibronenctin mRNA (by RT-PCR) and protein (by immunohistochemistry). The lungs of rats fed with ethanol showed increased fibronectin mRNA content when compared to pair-fed control animals (Figure 1A). As expected, immunohistochemical analysis of control lungs showed fibronectin deposition in vascular, airway structures, and within alveolar septae (Figure 1B). However, experimental lungs showed increased fibronectin deposition as demonstrated by intense staining of lung structures including the alveolar septae; both epithelial cells and interstitial fibroblasts appeared stained (Figure 1C). These observations demonstrate that chronic ethanol ingestion in rats is associated with increased expression and deposition of the matrix glycoprotein fibronectin *in vivo*.

Ethanol induces the expression of fibronectin in fibroblasts by stimulating the transcription of its gene.

Because of the increased deposition of fibronectin around fibroblasts in the lungs of ethanol-exposed rats, we explored the effects of ethanol in cultured lung fibroblasts with the intention of developing an *in vitro* model of ethanol-induced fibronectin expression. Using RT-PCR, we found that ethanol induced the expression of endogenous fibronectin mRNA in primary rat lung fibroblasts (Figure 2A). This coincided with an increase in fibronectin protein production as determined by western blotting (Figure 2B). These observations identify the lung fibroblast as a potential target for ethanol in the lung.

The data presented above also suggested that the effects of ethanol on fibronectin expression occur at the level of gene transcription. To test this, we used NIH3T3 fibroblasts which were considered a suitable model for our studies because they respond to ethanol in a similar fashion as primary lung fibroblasts. This is demonstrated in Figure 3A where NIH3T3

cells treated with ethanol (60 mM) show increased accumulation of endogenous fibronectin mRNA as determined by RT-PCR. The increase in fibronectin mRNA was detectable as early as 8 hours after ethanol stimulation. As expected, the ethanol-induced response was associated with a subsequent increase in fibronectin protein production as determined by western blotting that was highest at 48 hours (Figure 3B).

When the transcription of the gene was tested in NIH 3T3 cells stably transfected with a construct containing the human fibronectin promoter fused to a luciferase reporter gene, pFN(1.2kb)LUC, as previously reported (20), we found that ethanol increased the transcription of pFN(1.2kb)LUC when compared to control (Figure 4A-C). As before, the effects of ethanol were both time-dependent (peaking around 8 hours) and dose-dependent (optimal at 60 mM). At the concentrations used, ethanol had no effect on cell viability (not shown).

Ethanol-induction of fibronectin gene transcription is dependent on protein kinase activity and the induction of cAMP Response Element Binding Protein (CREB)

The stimulation of fibronectin by serum and other agents has been shown to be preceeded by protein kinase activation and phosphorylation of the transcription factor cAMP Response Element Binding Protein (CREB), followed by CREB binding to cAMP response elements (CREs) in the fibronectin promoter (20). To test whether this pathway also mediates the ethanol-induced fibronectin response, we performed the following experiments. First, we tested the effects of protein kinase inhibitors on the ethanol-induced response. As depicted in Figure 5A, a protein kinase C inhibitor, Calphostin C (25), abolished the constitutive expression of fibronectin as well as expression induced by ethanol. PD98059, an inhibitor of MEK-1 (26), which is upstream of erk-1 and erk-2 in the Mitogen Activation Protein Kinase pathway, also inhibited the ethanol-induced fibronectin response (Figure 5B). The inhibitory effects of these agents at the concentrations used were not associated with increased cell death (not shown).

Second, we tested the effects of ethanol on CREB. The exposure of fibroblasts to 60 mM ethanol induced the phosphorylation of CREB in a time-dependent fashion (Figure 6A). This event was associated with increased binding of CREB to DNA as determined by EMSA (Figure 6B). Note that a competing oligonucleotide abolished the binding of CREB, whereas the mutated oligonucleotide did not affect it.

Ethanol induction of fibronectin gene transcription is dependent on specific promoter elements.

The above studies suggested a role for specific promoter elements within the fibronectin gene, namely the CREs, in the ethanol-induced effect. To confirm this role, we tested fibroblasts transfected with deletion constructs of the fibronectin gene promoter. All previous experiments were performed using pFN(1.2kb)LUC. In the experiments described in Figure 7A, we tested cells transfected with pFN(0.5kb)LUC, that lacks most of the 5' sequences present in pFN(1.2kb)LUC, but contains the 3 CREs, or transfected with pFN(0.2kb)LUC, that lacks all three CREs. Ethanol stimulated the transcription of pFN(1.2kb)LUC over control as before. The stimulatory effect of ethanol was unaffected when the deletion construct pFN(0.5kb)LUC was tested indicating that 5' sequences proximal to the CREs are not needed for optimal stimulation. In contrast, no stimulation was noted for ethanol when all CREs were lacking.

These data suggest, but do not prove, a role for CREs in the ethanol-induced response. To strengthen the association between CREs and the response tested, we measured fibronectin expression in cells exposed to 60 mM ethanol after transfection with a competing consensus CRE oligonucleotide (Figure 7B). The consensus CRE oligonucleotide greatly diminished the ethanol-induced fibronectin response, whereas the control mutated CRE oligonucleotide had no effect.

Ethanol stimulates fibronectin gene transcription via α7 nAChR-dependent signals

Ethanol has been shown to act on nicotinic acetylcholine receptors (nAChRs) in neuronal cells (27). Because nAChRs have been detected in NIH3T3 fibroblasts and monkey lung fibroblasts (28), among other non-neuronal cells, we examined the role of these receptors in our system. First, we demonstrated that NIH3T3 cells express mRNA coding for α7 nAChRs (Figure 8A). Of note, the α7 mRNA was increased after ethanol stimulation. Further evidence for the presence of α7 nAChRs was derived from α-bungarotoxin- (α-BGT) binding assays. α-BGT is a specific competitive ligand for α7 nAChRs (27). Consistent with the expression of α7 nAChRs, we found binding sites for α-BGT on the surface of fibroblasts (Figure 8B). As expected, the binding of α-BGT was increased after the exposure of the cells to ethanol for 24 hours. This increase in ethanol induced α-BGT binding was decreased by excess unlabeled α-BGT and ethanol.

Together, these observations indicated that fibroblasts express α7 mRNA and have α7 nAChR protein on their surface. To confirm a role for α7 nAChRs in the ethanol-induced fibronectin response, we pretreated fibroblasts with α-BGT prior to exposing them to ethanol. As shown in Figure 8C, α-BGT completely prevented the expression of the fibronectin in response to ethanol.

The ethanol-induced fibronectin response is dependent on ethanol metabolism

In hepatic cells, ethanol induction of procollagen can be inhibited by 4-methylpyrazole, a blocker of alcohol dehydrogenase (29). This suggests that the ability of ethanol to stimulate matrix gene expression is dependent on its metabolism and conversion into aldehyde. To test this possibility in our system, NIH3T3 transfected fibroblasts were pre-treated with 4 methylpyrazole prior to stimulation with ethanol. As depicted in Figure 9A, this treatment inhibited the induction of fibronectin by ethanol. In contrast, 4-methylpyrazole did not affect the induction of fibronectin by nicotine, another ligand for α7 nAChRs (Figure 9B).

DISCUSSION

Mechanisms of ethanol induction of fibronectin in fibroblasts

This report demonstrates for the first time that chronic ethanol ingestion in animals results in increased expression of fibronectin mRNA and deposition of its protein in the lung. In addition, it identifies the lung fibroblast as a target for ethanol. Studies performed with cultured fibroblasts revealed that ethanol induces fibronectin gene transcription by acting on α7 nAChRs, and by inducing the phosphorylation and nuclear translocation of CREB, a key transcription factor capable of initiating fibronectin gene expression (20). Finally, the report demonstrates that ethanol-induction of fibronectin is dependent on ethanol metabolism, protein kinase activity, and specific transcriptional elements within the fibronectin gene promoter. Together, these studies suggest that ethanol can directly affect lung fibroblasts and induce their expression of fibronectin. We speculate that this may result in alterations in the composition of the lung matrix, and represents yet another potential mechanism by which ethanol renders the host susceptible to acute lung injury.

For years, it has been known that ethanol induces tissue remodeling in the liver where it can cause fibrosis and cirrhosis (30), but its effects on the lung have been poorly recognized until recently. In the liver, both ethanol and its metabolite acetaldehyde are considered to be fibrogenic and have been shown to induce the expression of collagen (31). Ethanol induces the production of collagen and the expression of α1(I)procollagen mRNA in fibroblasts and in primary cultures of liver stellate cells (32-34). Similar to our observations with fibronectin, the ethanol-induced collagen response in liver cells was found to be maximal with doses of ethanol between 50 and 100 mM, was optimal after 24-hour exposure, was dependent on protein synthesis, and appeared to occur at the level of gene transcription (32). In that system, as in ours, the response was abolished by an inhibitor of ethanol metabolism to acetaldehyde, 4-methylpyrazole, suggesting that ethanol metabolism was needed to observe its effects on collagen expression. Of interest, the induction of collagen by ethanol was detected in liver stellate cells, but not in primary cultures of hepatocytes suggesting that not all cells of an organ respond to ethanol equally.

Ethanol-induced fibronectin expression has also been demonstrated in the liver. Increased total and cellular fibronectin protein production was detected in the liver of rats

exposed to ethanol in their diet for 8-12 weeks (33). However, the intracellular pathways responsible for its induction and how they relate to fibronectin induction in pulmonary cells are unclear. Our studies show that ethanol induction of fibronectin is dependent on the activity of protein kinases such as Protein Kinase C and Mitogen-Activated Protein Kinases. This is reminiscent of the work of Svegliati-Baroni and colleagues who demonstrated that the stimulation of fibronectin expression in human hepatic stellate cells is associated with a time-dependent phosphorylation of pp70(S6K) and extracellular-regulated kinases-1 and -2. In their system, the stimulatory effect of ethanol was also inhibited by Calphostin C and PD98059 (34).

Our work also shows that ethanol-induction of fibronectin is dependent on the activation and DNA binding by CREB, an important modulator of fibronectin gene transcription (20,35,36). Fibronectin gene expression occurs rapidly in response to a variety of cytokines and growth factors (e.g., transforming growth factor β1), as well as changes in cell shape and attachment (36). The 5' sequences containing regulatory elements of the fibronectin gene have been cloned and characterized. In addition to a TATA box and several other transcriptional elements (i.e., NF-1, SP-1), the promoters of the human and murine fibronectin genes contain three cyclic AMP-response elements or CREs that appear to be the dominant regulators of fibronectin gene transcription (35,36). In our system, these CREs appear to be critical in mediating the effects of ethanol.

Role of nAChRs in ethanol-induced fibronectin expression

Perhaps the most significant finding of this work relates to the demonstration of α7 nAChRs in fibroblasts, and the confirmation that they mediate the ethanol-induction of fibronectin via specific intracellular signals. These observations are consistent with most other studies available to date in neuronal cells showing that ethanol acts mainly via nAChRs (37,38). nAChRs are a family of multimeric acetylcholine-triggered cation channel proteins that form the predominant excitatory neurotransmitter receptors on muscles and nerves in the peripheral nervous system. They are also expressed in lower amounts throughout the central nervous system. At least thirteen genes that code for nAChRs have been identified to date; four β subunits and nine α subunits. In each of these receptors, the various subunits assemble into pentamers in a homomeric or heteromeric fashion (39). The most abundant homomeric form is

$(\alpha 7)_5$. This is the receptor that our data with α-bungarotoxin points to as a mediator of the effects of ethanol in the lung.

Little is known about nAChR expression and function outside of the central and peripheral nervous systems. nAChRs have been demonstrated in immune cells (40), keratinocytes (41), and, consistent with our data, in NIH3T3 fibroblasts (41). Evidence for the expression of functional nAChRs in lung cells is also available. α7 nAChR subunits have been detected in both human and mouse bronchial epithelial cells, and in submucosal glands (42). α7 has also been reported in small cell lung cancer (SCLC) and SCLC cell lines, and the growth of these cells can be inhibited by α-bungarotoxin, a specific antagonist of α7 receptors (43). Others have demonstrated in primates that nicotine, by binding to specific nAChRs, can affect lung development (28). When they examined for nAChRs in control animals, they detected α7 predominantly in fibroblasts surrounding the walls of airways and vessels, among other cell types. The expression of this receptor increased dramatically in animals exposed to nicotine. This was associated with increased collagen deposition surrounding the cartilaginous large airways and vessels. Overall, our observations and those described above suggest that lung cells (in particular fibroblasts) express functional nAChRs and that, by binding to these receptors, ethanol (and other ligands such as nicotine), can affect tissue remodeling in lung.

It is important to note that ethanol is capable of inducing the expression of α7 nAChR mRNA and increase the binding of α-BGT at the cell surface, presumably due to increased α7 nAChR protein. This observation unveils a potential amplification mechanism for the effects of ethanol. By increasing the expression of nAChRs on fibroblasts, fibronectin expression in response to ethanol is further enhanced. In other work, we showed that ethanol increases the transcription of the gene coding for the α5 subunit of the α5β1 fibronectin integrin receptor (not shown). This could represent yet another mechanism of amplification for the effects of ethanol in the lung.

Another interesting observation relates to the ability of 4-methylpyrazole to inhibit the effects of ethanol on fibronectin expression, but not those of nicotine. Nicotine is considered a ligand for α7 nAChRs, and we have demonstrated that it is also capable of inducing fibronectin (unpublished observations). This observation is important because it suggests that these agents can induce fibronectin via distinct α7 nAChR-mediated signals. In the case of ethanol, it appears that signal transduction requires alcohol metabolism. This, and other observations, suggest that,

even though both ethanol and nicotine can stimulate nAChRs, they trigger different intracellular signals. This, together with differences in cell recognition and metabolism might explain why nicotine and ethanol abuse are associated with the development of different clinical entities.

Implications for our understanding of acute lung injury

The observation that ethanol can induce fibronectin expression in the lung is an important one because fibronectin deposition is increased in many, if not all, forms of clinical and experimental acute lung injury, and it has been implicated in the pathogenesis of this illness (13-15). Its exaggerated deposition under these circumstances has considerable effects on lung structure. For example, fibronectin promotes collagen deposition in connective tissue (44). In doing so, the newly deposited fibronectin-containing matrices provide a scaffold for the migration of epithelial cells across denuded basement membranes and the organization of immune cells and fibroblasts in extravascular spaces (45). Fibronectin also affects many cellular functions. It has been shown to promote the adhesion and migration, proliferation, and differentiation of many lung cell types including epithelial and endothelial cells and fibroblasts (13,14). With regards to immune cells, fibronectin has been shown to be chemotactic to monocytes and endothelial cells, among other cells (15), and to stimulate their expression of proinflammatory cytokines that, in turn, could amplify the inflammatory and repair responses of the lung after injury (46-49).

The biological effects of fibronectin are possible because of its ability to interact with specific cell surface integrin receptors capable of signal transduction (50). The activation of the integrin fibronectin receptor $\alpha5\beta1$ elicits the activation of intracellular signals including increased cAMP levels, calcium fluxes, and the activation of protein kinases. These events lead to the induction of potent transcription factors including Activator Protein-1 and Nuclear Factor kappa B (47-49) that control the transcription of many genes including the proinflammatory cytokines interleukin-1β and tumor necrosis factor-α and the vascular cell adhesion molecule-1.

In view of the above, it is postulated that the exaggerated deposition of fibronectin in the lungs of ethanol-treated animals alters the composition of the lung extracellular matrix. This response is amplified by ethanol's ability to increase the expression of $\alpha7$ nAChRs in fibroblasts that mediate the fibronectin induction. In turn, the newly deposited fibronectin-containing matrix primes lung resident and incoming cells to respond to injurious agents in an exaggerated

manner. In doing so, fibronectin promotes the development of an aggressive uncontrolled tissue remodeling and inflammatory response that leads to tissue destruction rather than repair after injury. Further delineation of the factors and conditions that regulate ethanol-induced fibronectin expression in the lung will be needed before a full understanding can be obtained of the true consequences this process has in the lung.

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FIGURE LEGENDS

Figure 1. Increased fibronectin mRNA (A) and protein (B) in the lungs of ethanol-treated rats.

A, Induction of fibronectin mRNA in lung. Sragues-Dawley rats were fed for 6 weeks with the Lieber-DeCarli liquid diet with ethanol (n=5; 36% total calories) or with an isocaloric substitution with Maltin-Dextrin (n=5; control diet). Afterwards, the lungs were perfused, harvested *en-bloc*, and processed for mRNA isolation and RT-PCR analysis to evaluate message for endogenous fibronectin. Relative fibronectin mRNA values were normalized to HPRT and shown as mean ± SD. Note that lungs from ethanol-fed rats contained higher levels of fibronectin mRNA.

B, Distribution of fibronectin protein in control lungs. The lungs from control animals were fixed, processed, sectioned, and submitted to immunohistochemistry with an anti-fibronectin polyclonal antibody (FN-1). Note that staining for fibronectin was detected around vascular and airway structures. Fibronectin staining was also detected within the alveolar epithelium and within the alveolar septae (insert).

C, Distribution of fibronectin protein in ethanol-treated animals. The lungs from ethanol-fed rats were fixed, processed, sectioned, and submitted to immunohistochemistry with an anti-fibronectin polyclonal antibody (FN-1) as described above. As in the control, fibronectin staining was detected around vascular and airway structures. It was also detected in the alveolar epithelium and within alveolar septae (insert).

Figure 2. Ethanol induces the synthesis of fibronectin in rat lung fibroblasts.

A, Induction of fibronectin mRNA. Rat lung fibroblasts were cultured in the presence of physiological concentrations of ethanol (60 mM) for 24 hours after which they were harvested and the cell extracts processed for RT-PCR analysis of fibronectin mRNA. Relative fibronectin mRNA values were normalized to HPRT and shown as mean ± SD. Note that ethanol induced fibronectin mRNA accumulation when compared to control.

B, Induction of fibronectin protein. Fibroblasts were cultured as described above for up to 48 hours. Afterwards, the cell extracts were tested for fibronectin protein by western blotting. The data is depicted as densitometric units obtained from analysis of a representative western blot gel. Ethanol induced fibronectin production at both 24 and 48 hours.

Figure 3. Ethanol induces endogenous fibronectin production in NIH3T3 fibroblasts.

A, Induction of fibronectin mRNA. Fibroblasts were cultured in the presence of ethanol (60 mM) for up to 24 hours. Afterwards, the cells were harvested and processed for RT-PCR to detect fibronectin mRNA. Note that ethanol induced the accumulation of fibronectin mRNA by 8 hours and this persisted at 24 hours. Data is presented as mean ± SD.

B, Induction of fibronectin protein. Fibroblasts were treated with ethanol (60 mM) as described above, harvested for western blotting, and fibronectin was detected using an anti-fibronectin antibody (FN 1.2; 1:500 dilution). Note that ethanol induced the production of fibronectin protein by 24 hours. Data is presented as mean ± SD.

Figure 4. Ethanol stimulates fibronectin gene transcription in NIH3T3 fibroblasts.

A, Stimulation of fibronectin gene transcription. NIH 3T3 fibroblasts stably transfected with the fibronectin-luciferase promoter construct were treated with ethanol (60 mM) for 24 hours. Afterwards, the cells were harvested and processed to assess fibronectin gene transcription by measuring luminescence and the data is presented as mean ± SD. Note that ethanol doubled the transcription of the fibronectin gene over control.

B, Dose-dependent stimulation of fibronectin gene transcription. Stably transfected fibroblasts were treated with ethanol ranging from 20 to 160 mM for 24 hours. The cells were harvested and fibronectin gene transcription was quantified by measuring luminescence. Data is presented as mean ± SD.

C, Time-dependent stimulation of fibronectin gene transcription. Stably transfected fibroblasts were treated with 60 mM ethanol from 2 to 48 hours. Afterwards, cells were harvested and fibronectin gene transcription was quantified by measuring luminescence. Data is presented as mean ± SD.

Figure 5. Ethanol induces fibronectin production via protein kinases.

A, Protein kinase C-dependent production of fibronectin. Transfected fibroblasts were cultured with ethanol (60 mM) for 16 hours in the presence and absence of the protein kinase C inhibitor Calphostin C (1×10^{-7} M). Data is presented as mean ± SD. Note that activated Calphostin C

(ACC) inhibited the ethanol stimulated expression of the gene. The inactive reagent had no effect (not shown).

B, Mitogen Activated Protein Kinase-dependent production of fibronectin. Transfected fibroblasts were cultured as before in the presence of ethanol (60 mM) with or without the MEK-1 inhibitor PD98059 (50 uM) for 16 hours. Afterwards, fibronectin gene transcription was measured and data is presented as mean ± SD. Note that the MEK-1 inhibitor prevented the stimulatory effect of ethanol, but it did not block the constitutive expression of the gene.

Figure 6. Ethanol stimulates CREB phosphorylation and DNA binding.

A, Induction of CREB phosphorylation. NIH 3T3 fibroblasts treated with ethanol (60 mM) for 0-24 hours were processed for western blotting with an antibody to phosphorylated CREB (serine 133). Note that ethanol induced the phosphorylation of CREB and ATF-1, most noticeably by 2-4 hours.

B, Induction of CREB DNA binding. Fibroblasts were left untreated or treated with ethanol (60 mM) for 16 hours. Afterwards, they were processed for EMSA to detect CREB. Note that ethanol induced DNA binding by CREB (lane 3) when compared to control (lane 2). This induction was inhibited with a non-labeled consensus double-stranded CREB oligonucleotide (50x excess; lane 4), but not by a mutated double-stranded CREB oligonucleotide (lane 5). Bound, DNA-protein complex.

Figure 7. Promoter elements involved in ethanol-induced fibronectin expression

A, Experiments with deletion mutants. Fibroblasts were treated with ethanol (60 mM) as described above after transfection with the full-length construct, pFN(1.2kb)LUC, or one of the two deletion constructs: pFN(0.5kb)LUC contains all 3 CREs, but lacks much of the 5' sequences, whereas pFN(0.2kb)LUC lacks all 3 CREs. Data is presented as mean ± SD. Note that the pFN(0.2kb)LUC construct did not respond optimally.

B, Inhibition by cotransfection with CREB oligonucleotide. Stably transfected fibroblasts were co-transfected with a competing consensus double-stranded CREB oligonucleotide (20 ug) or double-stranded mutated oligonucleotide (mCREB) (20 ug) by electroporation followed by exposure to ethanol (60 mM) for 16 hours. Afterwards, the cells were harvested and processed to assess fibronectin gene transcription by measuring luminescence and data is presented as

mean ± SD. Note that the competing CREB oligonucleotide inhibited the stimulatory effect of ethanol. The mutated oligonucleotide (mCREB) had no effect.

Figure 8. Ethanol stimulates the expression of α7 nAChRs in fibroblasts.

A, Induction of α7 nAChR mRNA. NIH 3T3 fibroblasts were exposed to ethanol as described before for 0-12 hours. Afterwards, the cells were harvested and processed for RT-PCR to detect α7 nAChR mRNA. Data is presented as mean ± SD. Note that ethanol induced the accumulation of α7 mRNA by 4 hours and this effect persisted by 12 hours.

B, α-bungarotoxin (α-BGT) binding assay. NIH 3T3 fibroblasts were cultured alone or in the presence of ethanol (60 mM) for 16 hours. Afterwards, they were incubated with ^{125}I-α-BGT, washed, and radioactivity counts measured. Excess competing non-radiolabeled α-BGT was added in the presence or absence of ethanol to determine specificity of binding. Data is presented as mean ± SD. Note that unstimulated cells expressed little α-BGT-binding sites. In contrast, the binding of α-BGT was increased in ethanol-treated cells. The unlabeled α-BGT diminished the ethanol induced ^{125}I-α-BGT binding.

C, Inhibition of fibronectin expression by α-bungarotoxin. Stably transfected fibroblasts were treated with ethanol (60 mM) for 24 hours in the presence or absence of α-BGT. Afterwards, the cells were harvested and processed to assess fibronectin gene transcription by measuring luminescence. Data is presented as mean ± SD. Note that α-BGT completely abolished the induction of fibronectin by ethanol.

Figure 9. Ethanol stimulation of fibronectin is inhibited by 4-methylpyrazole.

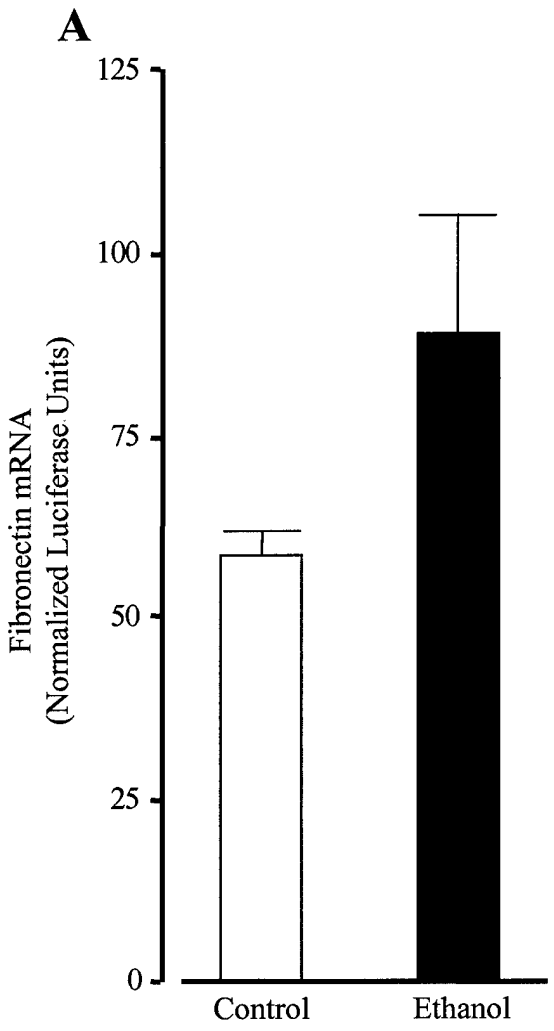
A, Effects on ethanol-induced fibronectin expression. Stably transfected fibroblasts were treated with ethanol (60 mM) in the presence or absence of 4-methylpyrazole (4-MP, 100 uM). Afterwards, the cells were harvested for the detection of fibronectin gene transcription via luminescence measurements. Data is presented as mean ± SD. Note that 4-MP inhibited the ethanol-induced response.

B, Effects on nicotine-induced fibronectin expression. Stably transfected fibroblasts were treated with nicotine (50 ug/ml) in the presence or absence of 4-methylpyrazole (4-MP, 100 uM). Afterwards, the cells were harvested for the detection of fibronectin gene transcription via

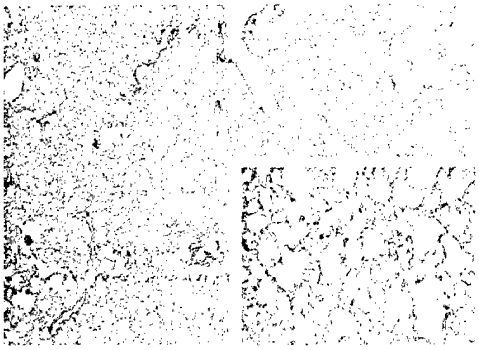
luminescence measurements. Data is presented as mean ± SD. Note that, in contrast to the studies with ethanol, 4-MP did not inhibit the nicotine-induced response.

Expression of FN mRNA by RT-PCR in Lung Tissue of Ethanol Fed Rats (n=5)

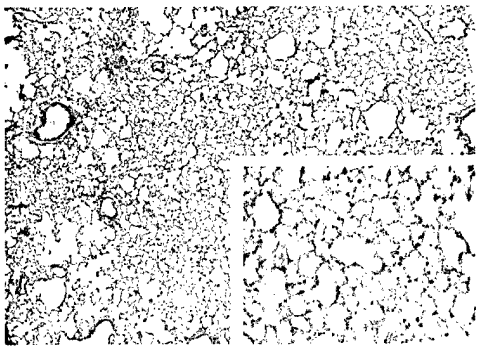
Roman: Figure 1A



B - Control

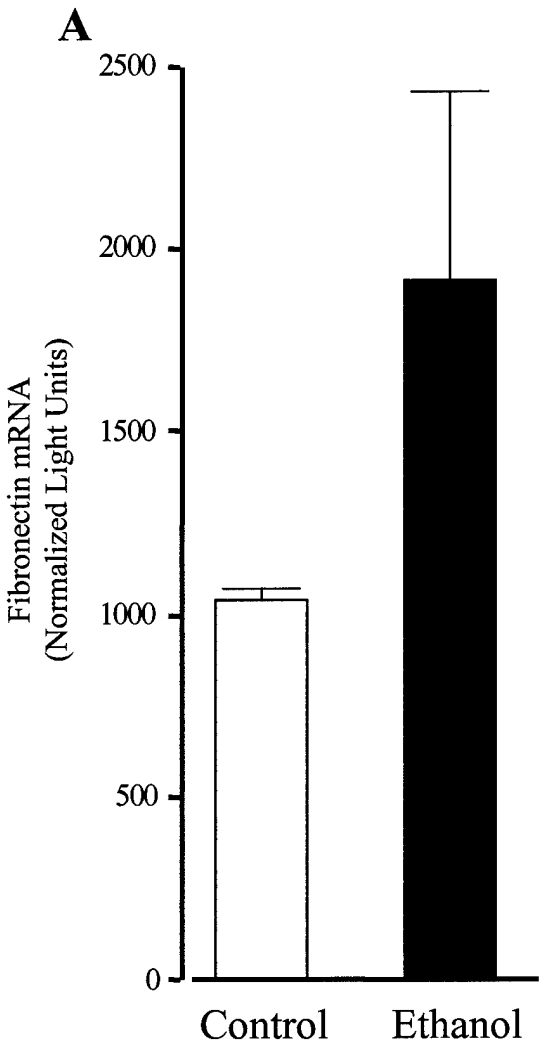


C - Ethanol



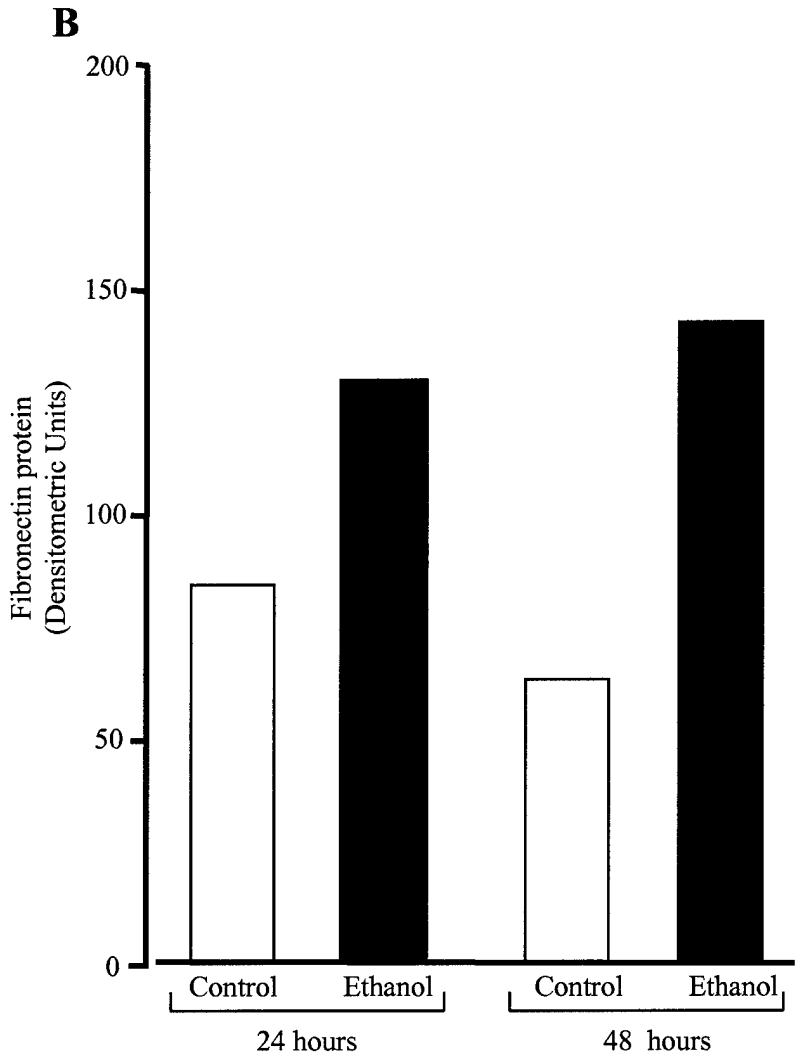
Roman: Figure 2A

Expression of FN mRNA by RT-PCR in Isolated Primary Lung Fibroblasts from Ethanol treated Rats



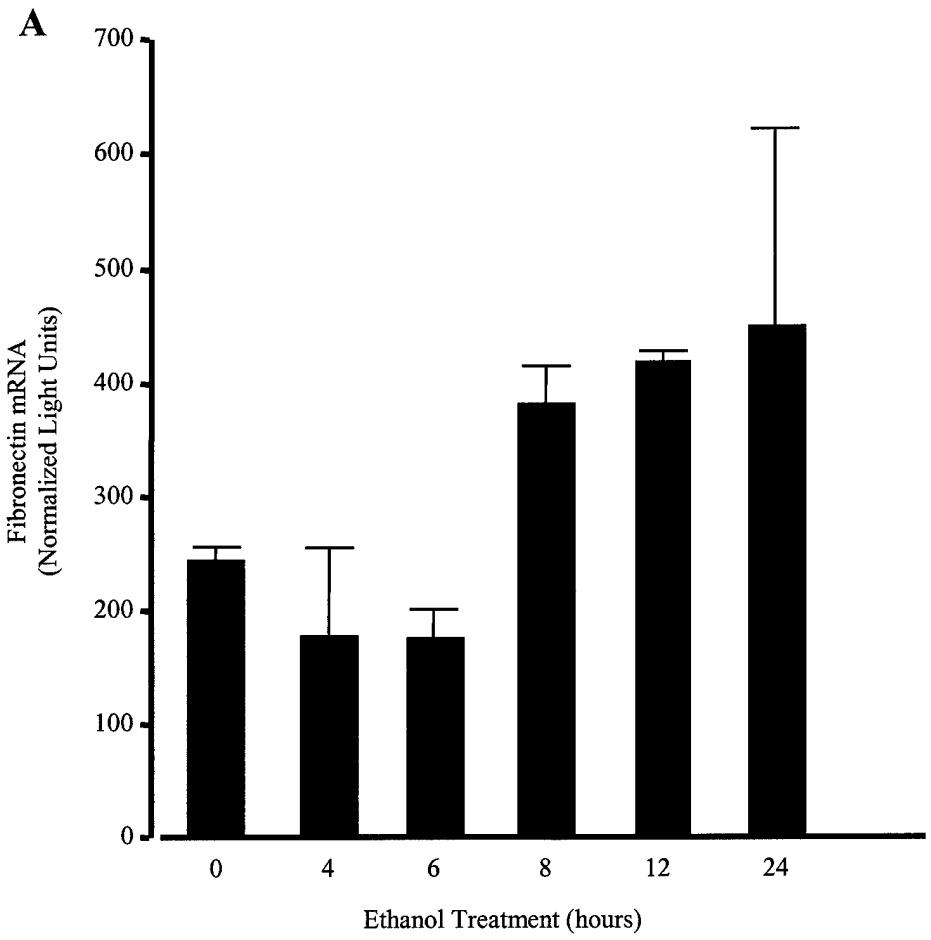
Roman: Figure 2B

FN Protein in Primary Rat Tracheal Fibroblasts:
Western Blot Densitometry



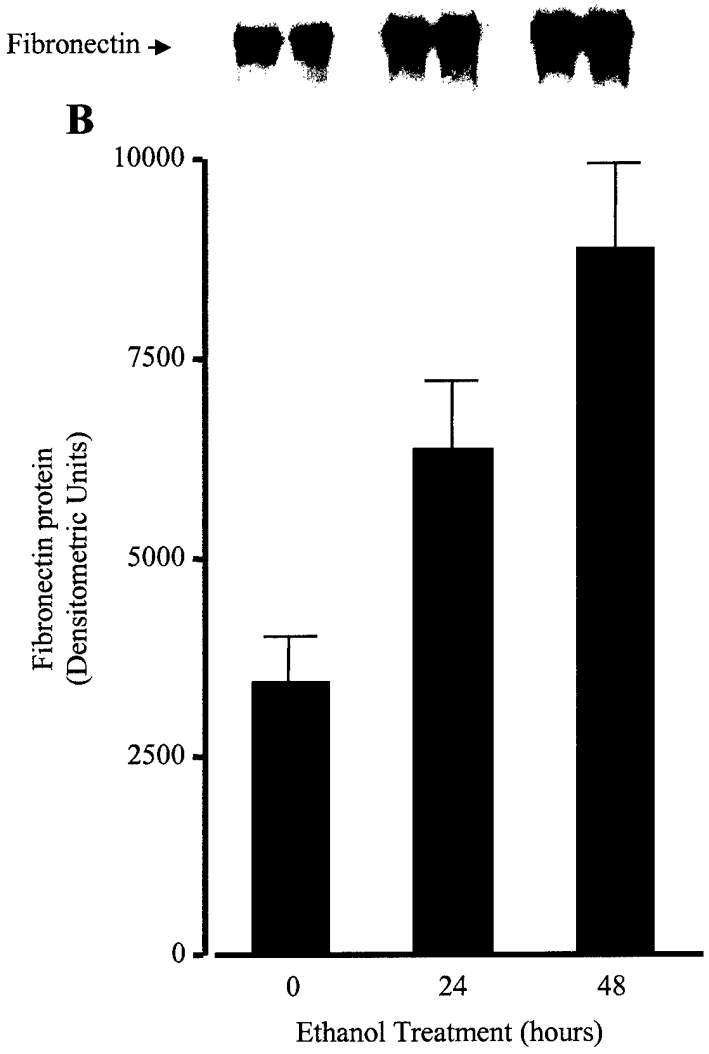
Expression of FN mRNA by RT-PCR

Roman: Figure 3A



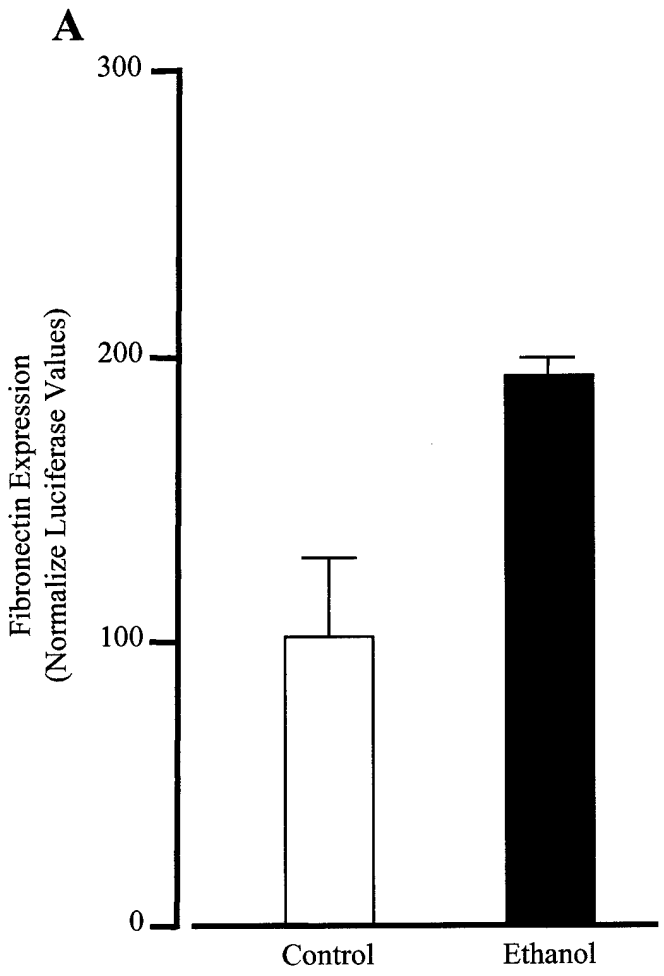
Expression of FN Protein

Roman: Figure 3B



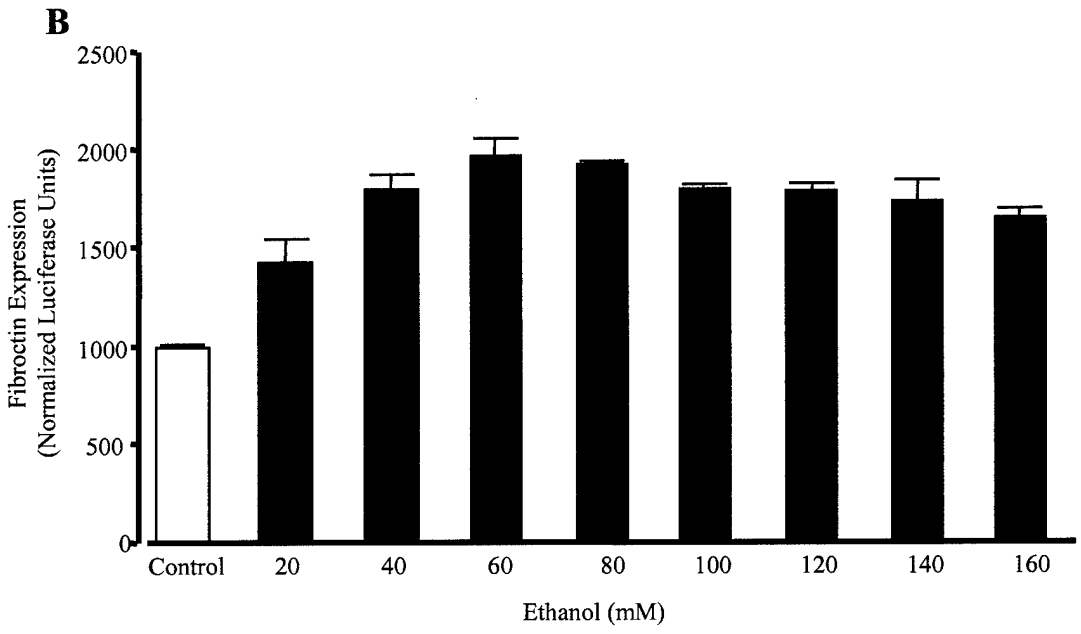
Roman: Figure 4A

Expression of Fibronectin Gene in NIH3T3 fibroblasts



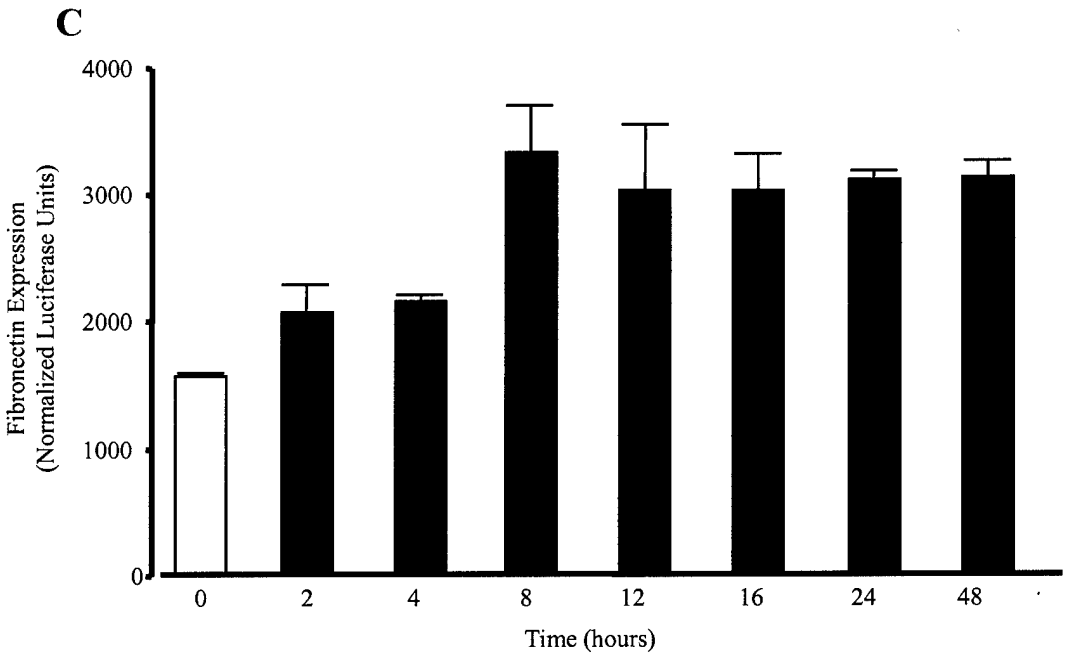
Expression of FN Gene in 3T3 fibroblasts: Dose Response

Roman: Figure 4B

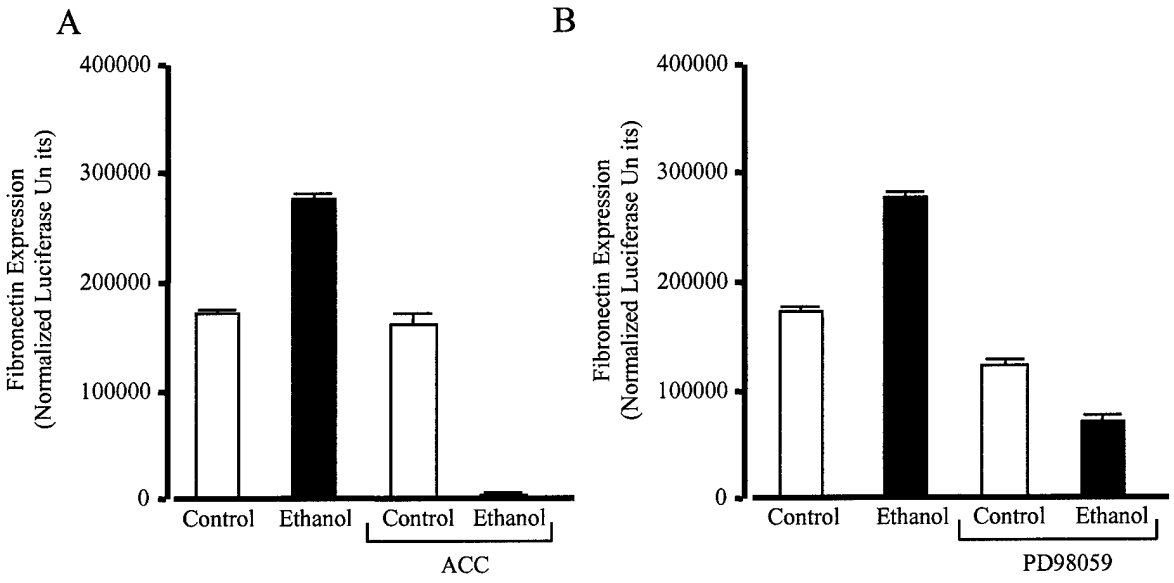


Expression of FN Gene in 3T3 fibroblasts: Time Study

Roman: Figure 4C



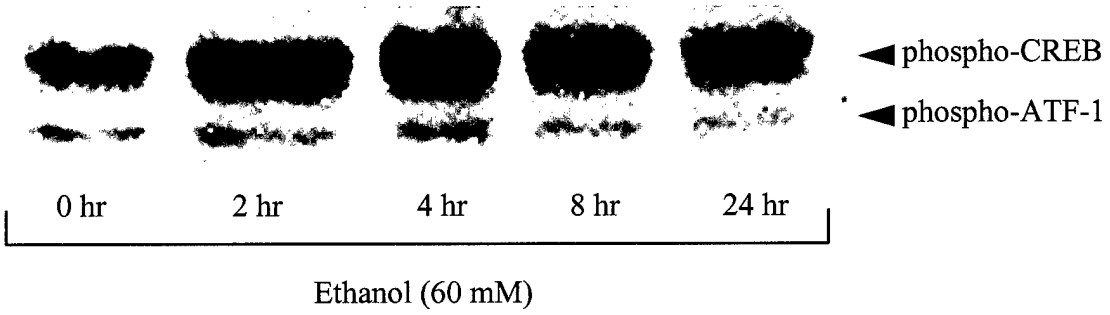
Expression of FN Gene in 3T3 fibroblasts: Effects of Calphostin C and MI Inhibitor Roman: Figure 5 A & B



Ethanol Stimulates CREB Phosphorylation in NIH3T3 fibroblasts

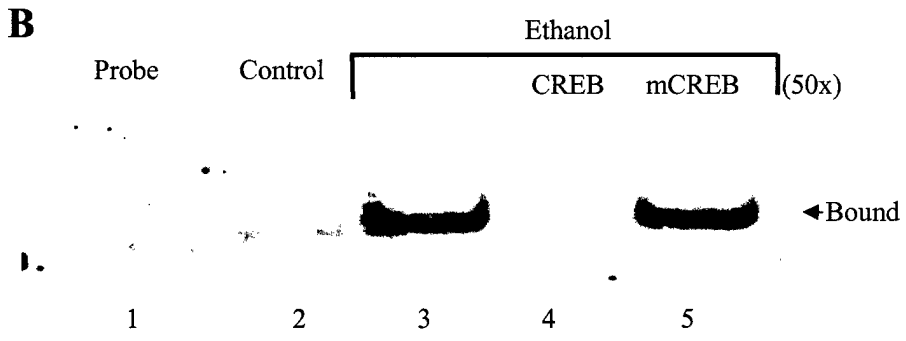
Roman: Figure 6A

A

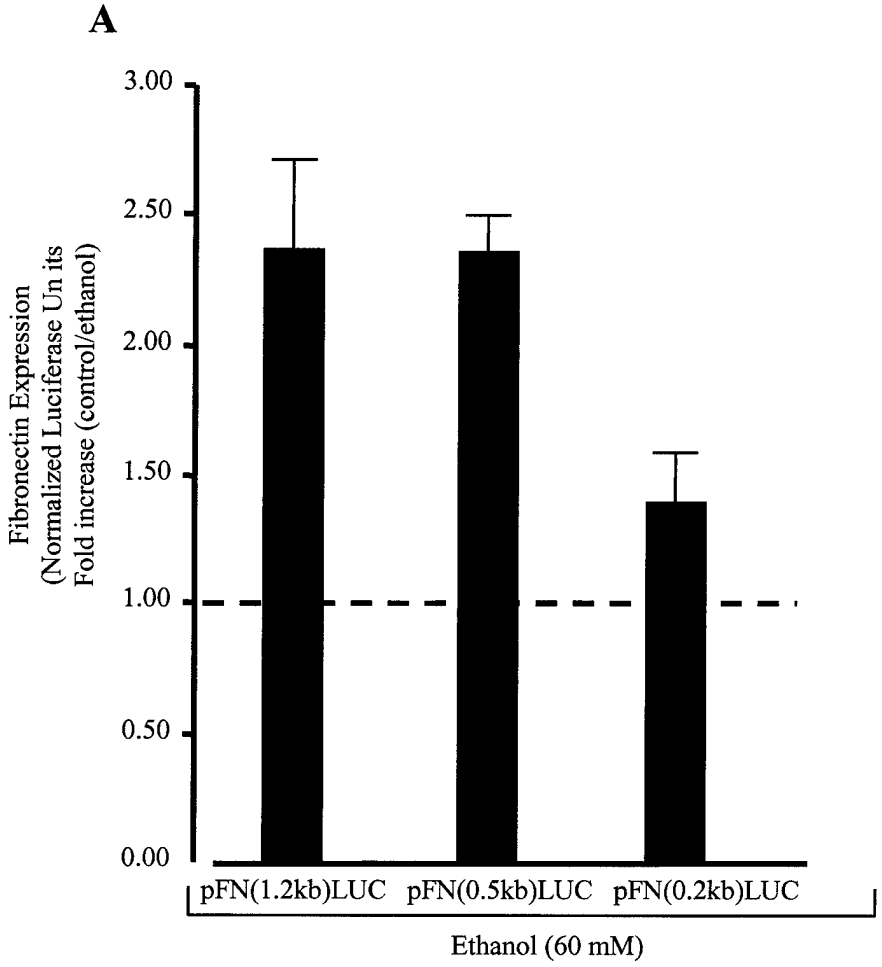


Ethanol increases DNA:CREB Nuclear Binding in NIH3T3 Fibroblasts

Roman: Figure 6B

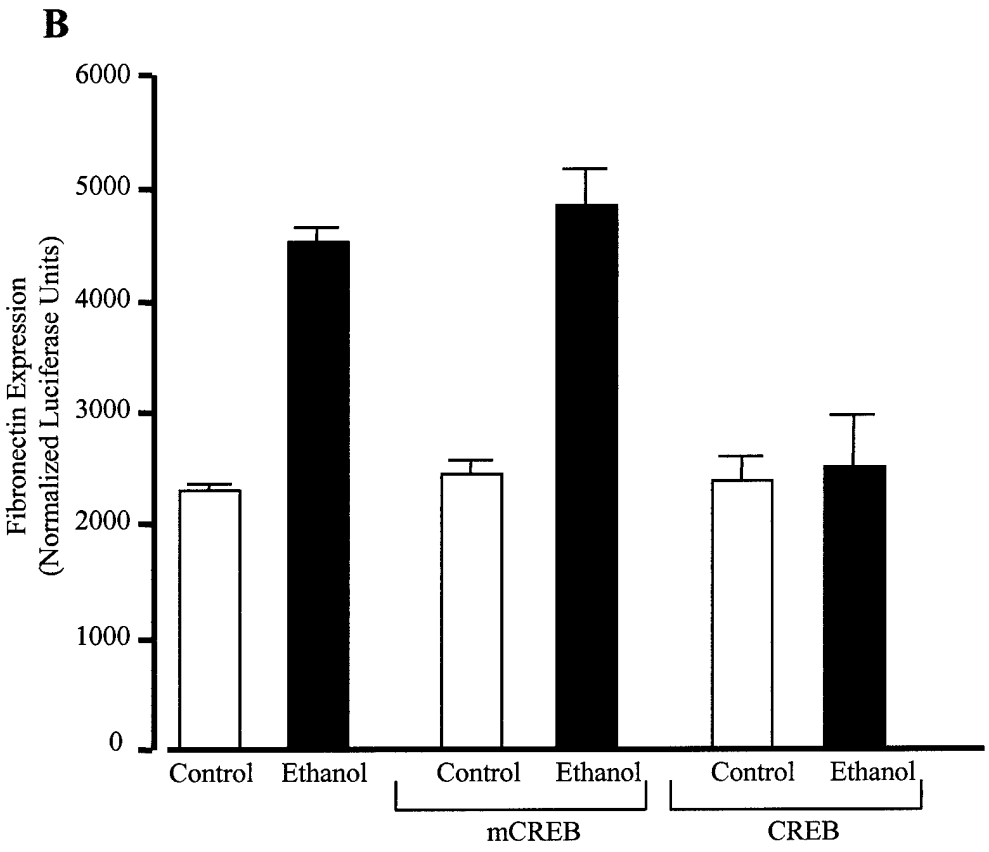


Roman: Figure 7A



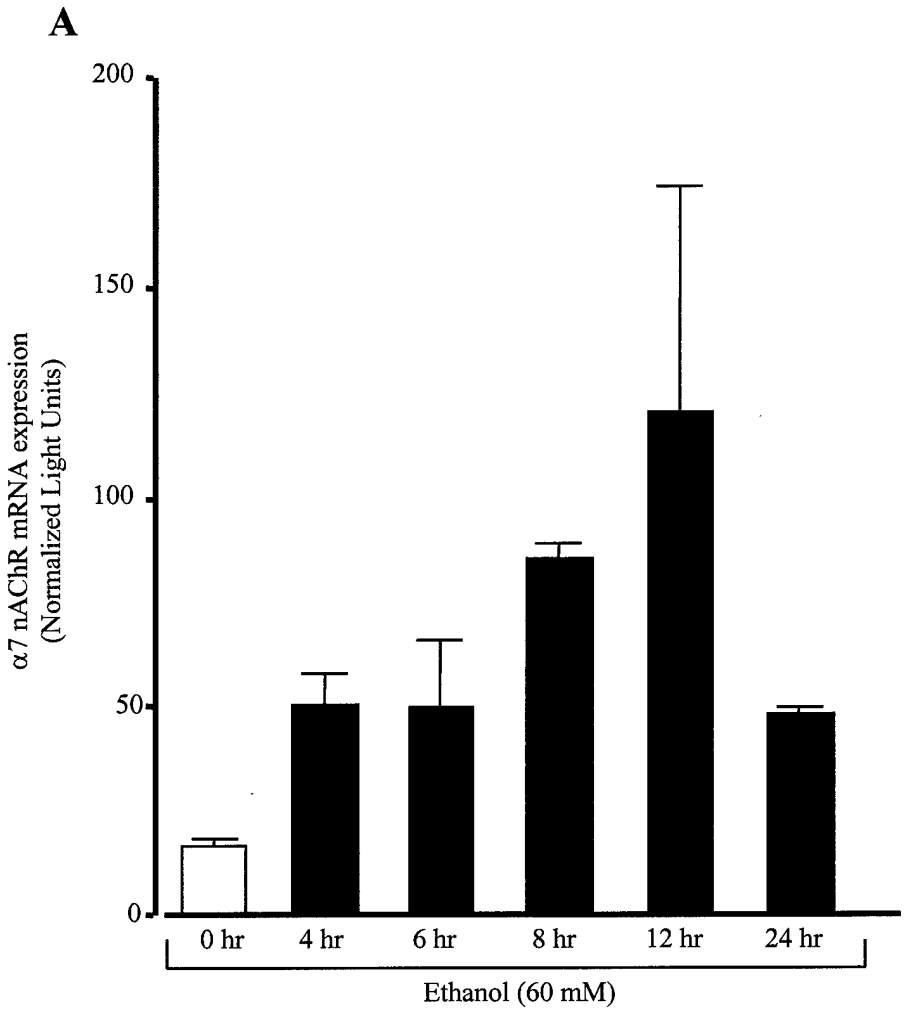
Roman: Figure 7B

Co-Transfection of NIH3T3 cells with CREB or mCREB Oligonucleotide:
Effect on Ethanol Induction of the Fibronectin Promoter



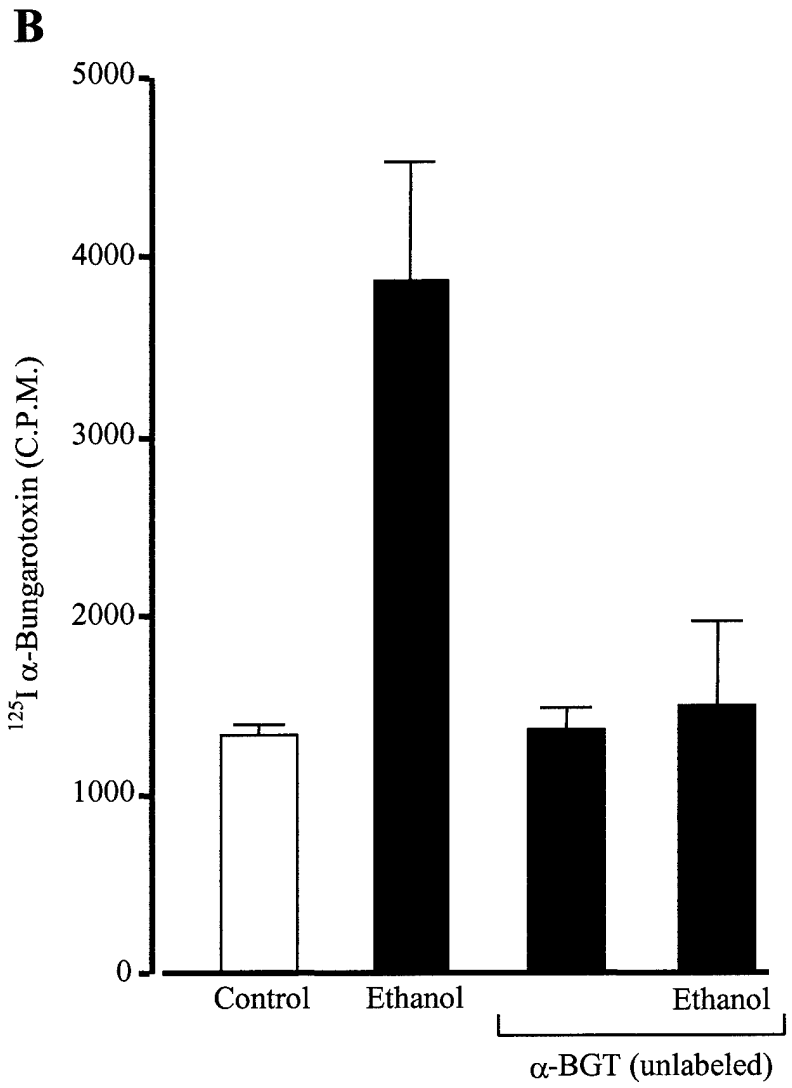
Ethanol Stimulation of the α subunit of nAChR receptors

Roman: Figure 8A



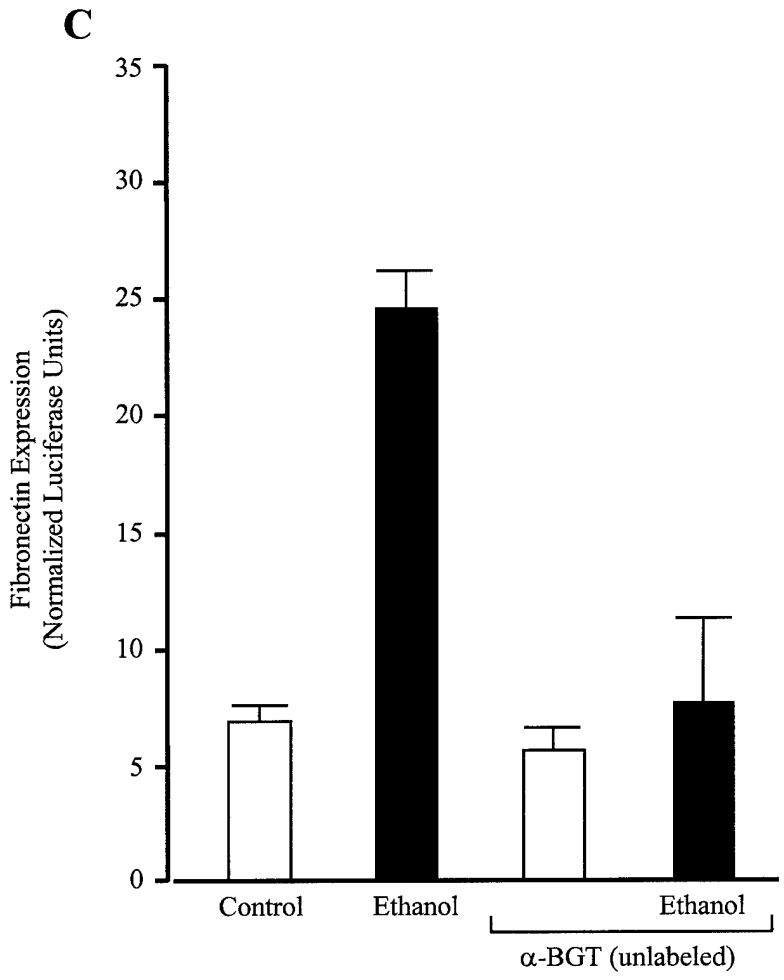
Increase in α-bungarotoxin binding with ethanol treatment

Roman: Figure 8B



Alpha Bungarotoxin inhibits Fibronectin Gene Transcription

Roman: Figure 8C



Ethanol stimulation of fibronectin is inhibited by 4-methylpyrazole

Roman: Figure 9 A & B